

presence or amount of said triple stranded complex as an indication of the presence or amount of nucleic acid A; wherein said triple stranded complex is more thermostable than a triple stranded complex formed from either of: (a) two nucleic acid A binding probes B binding with one nucleic acid A; or (b) two of said nucleic acid A binding probes C binding with one nucleic acid A.

87. The method according to claim 86 wherein said nucleic acid A binding probe C has only one part.

88. The method according to claim 86 wherein said nucleic acid A binding probe B comprises a binding region of 4 to 10 bases.

89. The method according to claim 86 wherein said binding region of nucleic acid A binding probe B has an asymmetric base sequence.

90. The method according to claim 86 wherein said binding region of nucleic acid A binding probe B has a symmetric base sequence.

91. The method according to claim 86 wherein said binding region of nucleic acid A binding probe C has a length of at least 6 bases.

RECEIVED

JUL 03 2000

92. The method according to claim 86 wherein said triple stranded complex comprises two different nucleic acid A binding probe C parts which bind to different regions of nucleic acid A in the triple stranded region.

93. The method according to claim 92 wherein said two different nucleic acid A binding probe C parts form a binding region which is comprised of two distinct triple helical binding regions, wherein each distinct triple helical binding region is formed from the binding of the two different probe C parts each to a distinct, non-overlapping, region on nucleic acid A.

94. The method according to claim 93 wherein said two different nucleic acid A binding probe C parts bind to adjacent regions on nucleic acid A.

95. The method according to claim 93 wherein said triple stranded complex is at least six bases in length and each of the two different nucleic acid A binding probe C parts individually contribute at least one but less than eleven bases to said triple stranded complex.

96. The method according to claim 86 wherein said binding region of nucleic acid A binding probe B comprises only pyrimidine bases but the binding region of the one or more nucleic acid A binding probe C parts comprises at least one non-pyrimidine base.

97. The method according to claim 86 wherein said nucleic acid A binding probe B is bound to nucleic acid A via Hoogsteen base pairing and the one or more nucleic acid A binding probe C parts are bound to nucleic acid A via Watson Crick base pairing.

98. The method according to claim 86 wherein at least one of said nucleic acid A binding probes is labeled and the presence of said label in the triple stranded complex is used for determining the presence or amount of the nucleic acid A.

99. The method according to claim 87 wherein at least one of said nucleic acid A binding probes has been chemically modified to destabilize triple helix formation occurring by either of: (a) two nucleic acid A binding probes B binding to one nucleic acid A; or (b) two of said nucleic acid A binding probes C binding with one nucleic acid A.

100. The method according to claim 86 wherein a first nucleic acid not to be determined is differentiated from said nucleic acid A by a difference in the base sequence located outside the binding region of the nucleic acid A binding probe B but within the binding region of the one or more nucleic acid A binding probe C parts.

101. The method according to claim 86 wherein a first nucleic acid not to be determined is differentiated from said nucleic acid A by a difference in the base sequence located within the binding region of the nucleic acid A binding probe B.

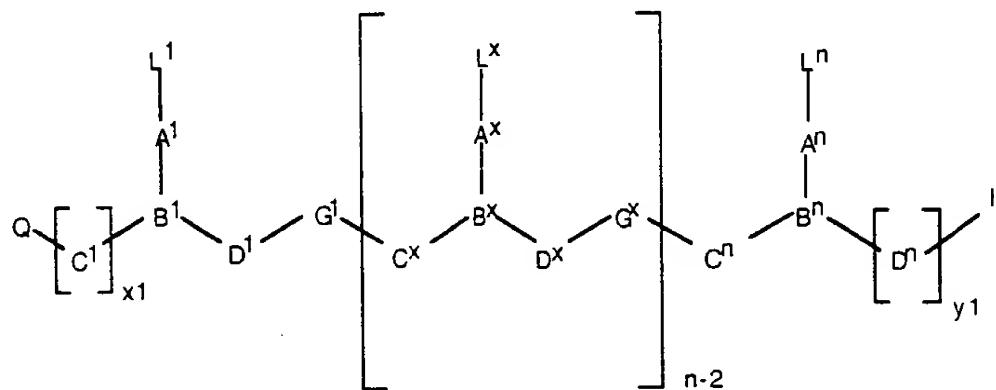
102. The method according to claim 86 wherein a reaction mixture is used for forming the triple stranded complex, said reaction mixture containing a competitive probe D which can

compete with at least one nucleic acid A binding probe C in binding to nucleic acid A, but which is incapable of participating in the formation of the triple stranded complex.

103. The method according to claim 86 wherein at least one of said nucleic acid A binding probes is a nucleic acid analogue.

104. The method according to claim 103 wherein said nucleic acid analogue is a peptide nucleic acid.

105. The method according to claim 86 wherein at least one of said nucleic acid A binding probes is a polymer of the general Formula I



Formula I

wherein

n is an integer of from at least 3,

x is an integer of from 2 to n-1,

each of  $L^1-L^n$  is a ligand independently selected from the group consisting of hydrogen, hydroxy,  $C_1-C_4$  alkanoyl, naturally occurring nucleobases, non-naturally occurring nucleobases, aromatic moieties, DNA intercalators, nucleobase-binding groups, heterocyclic moieties, reporter ligands and chelating moieties, wherein at least one of  $L^1-L^n$ , preferably at least one of  $L^2-L^{n-1}$  is a non-nucleobase electron acceptor or a donor moiety and at least 2 of  $L^1-L^n$  being a nucleobase binding group, or a naturally or non-naturally occurring nucleobase;

each of  $C^1-C^n$  is  $(CR^6R^7)_y$  (preferably  $CR^6R^7$ ,  $CHR^6CHR^7$  or  $CR^6R^7CH_2$ ) where  $R^6$  is hydrogen and  $R^7$  is selected from the group consisting of the side chains of naturally occurring alpha amino acids, or  $R^6$  and  $R^7$  are independently selected from the group consisting of hydrogen,  $(C_1-C_6)$  alkyl, aryl, aralkyl, heteroaryl, hydroxy,  $(C_1-C_6)$  alkoxy,  $(C_1-C_6)$  alkylthio,  $NR^3R^4$  and  $SR^5$ , where  $R^3$  and  $R^4$  are as defined below, and  $R^5$  is hydrogen,  $(C_1-C_6)$  alkyl, hydroxy,  $(C_1-C_6)$  alkoxy, or  $(C_1-C_6)$  alkylthio-substituted  $(C_1-C_6)$  alkyl or  $R^6$  and  $R^7$  taken together complete an alicyclic or heterocyclic system; or  $C^1-C^n$  is  $CO$ ,  $CS$ ,  $CNR^3$ ;

each of  $D^1-D^n$  is  $(CR^6R^7)_z$  (preferably  $CR^6R^7$ ,  $CHR^6CHR^7$  or  $CH_2CR^6R^7$ ) where  $R^6$  and  $R^7$  are as defined above;

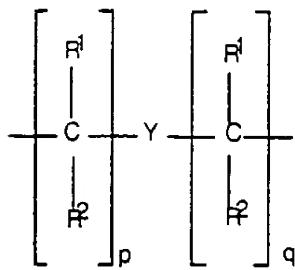
each of  $y$  and  $z$  is zero or an integer from 1 to 10, the sum  $y + z$  being at least 2, preferably greater than 2, but not more than 10;

each of  $G^1-G^{n-1}$  is  $-NR^3CO-$ ,  $-NR^3CS-$ ,  $-NR^3SO-$  or  $-NR^3SO^{2-}$ , in either orientation, where  $R^3$  is as defined below;

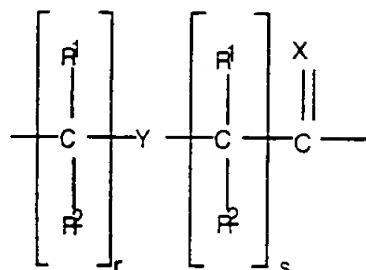
each of  $A^1-A^n$  and  $B^1-B^n$  are selected such that:

(a)  $A^1-A^n$  is a group of formula (I/A), (I/B), (I/C) or (I/D), and  $B^1-B^n$  is N or  $R^3N^+$ ; or

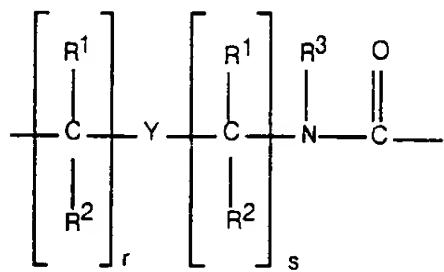
(b)  $A^1-A^n$  is a group of formula (I/D) and  $B^1-B^n$  is CH;



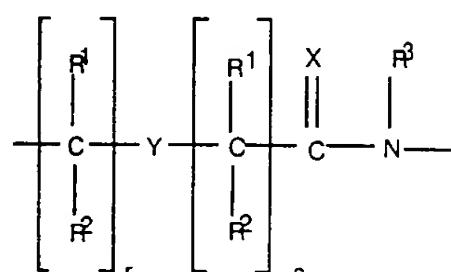
Formula I/A



Formula I/B



Formula I/C



Formula I/D

wherein:

$X$  is O, S, Se,  $NR^3$ ,  $CH_2$  or  $C(CH_3)_2$ ;

$Y$  is a single bond, O, S or  $NR^4$ ;

each of  $p$  and  $q$  is zero or an integer from 1 to 5, (the sum  $p+q$  being preferably not more than 5);

each of r and s is zero or an integer from 1 to 5, (the sum r+s being preferably not more than 5);

each R<sup>1</sup> and R<sup>2</sup> is independently selected from the group consisting of hydrogen, (C<sub>1</sub>-C<sub>4</sub>)alkyl which may be hydroxy- or (C<sub>1</sub>-C<sub>4</sub>)alkoxy- or (C<sub>1</sub>-C<sub>4</sub>)alkylthio-substituted, hydroxy, (C<sub>1</sub>-C<sub>4</sub>)alkoxy, (C<sub>1</sub>-C<sub>4</sub>)alkylthio, amino and halogen; and

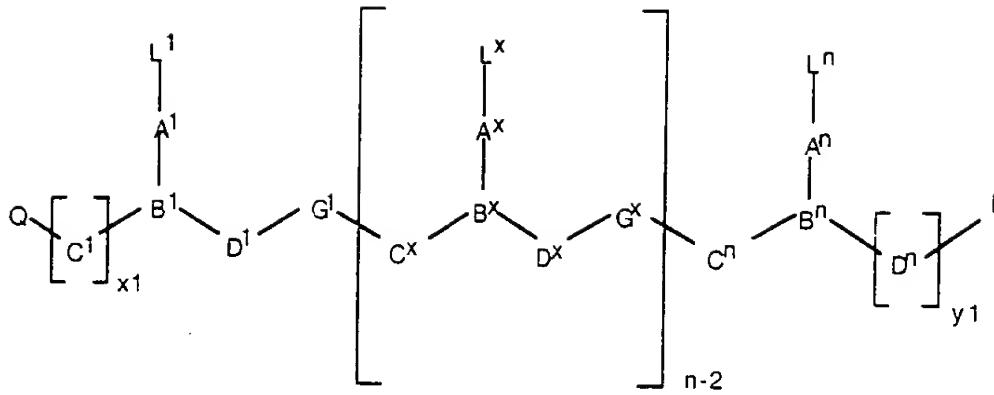
each R<sup>3</sup> and R<sup>4</sup> is independently selected from the group consisting of hydrogen, (C<sub>1</sub>-C<sub>4</sub>)alkyl, hydroxy- or alkoxy- or alkylthio-substituted (C<sub>1</sub>-C<sub>4</sub>)alkyl, hydroxy, (C<sub>1</sub>-C<sub>6</sub>)-alkoxy, (C<sub>1</sub>-C<sub>6</sub>)-alkylthio and amino;

Q and I is independently selected from -CO<sub>2</sub>H, -CONR'R'', -SO<sub>3</sub>H or -SO<sub>2</sub>NR'R'' or an activated derivative of -CO<sub>2</sub>H or -SO<sub>3</sub>H and -NR'R'''

where R', R'' and R''' are independently selected from the group consisting of hydrogen, alkyl, amino protecting groups, reporter ligands, intercalators, chelators, peptides, proteins, carbohydrates, lipids, steroids, nucleosides, nucleotides, nucleotide diphosphates, nucleotide triphosphates, oligonucleotides, including both oligoribonucleotides and oligodeoxyribonucleotides, oligonucleosides and soluble and non-soluble polymers and as well as nucleic acid binding moieties and

each of x<sub>1</sub> and y<sub>1</sub> is an integer of from 0 to 10.

106. A method according to claim 86 wherein at least one of said nucleic acid A binding probes is a polymer of the general Formula I



Formula I

wherein

n is an integer of from at least 3,

x is an integer of from 2 to n-1,

each of  $L^1-L^n$  is a ligand independently selected from the group consisting of hydrogen, hydroxy,  $C_1-C_4$  alkanoyl, naturally occurring nucleobases, non-naturally occurring nucleobases, aromatic moieties, DNA intercalators, nucleobase-binding groups, heterocyclic moieties, reporter ligands and chelating moieties, wherein at least one of  $L^1-L^n$ , preferably at least one of  $L^2-L^{n-1}$  is a non-nucleobase electron

acceptor or a donor moiety and at least 2 of L<sup>1</sup>-L<sup>n</sup> being a nucleobase binding group, or a naturally or non-naturally occurring nucleobase;

each of C<sup>1</sup>-C<sup>n</sup> is (CR<sup>6</sup>R<sup>7</sup>)<sub>y</sub> (preferably CR<sup>6</sup>R<sup>7</sup>, CHR<sup>6</sup>CHR<sup>7</sup> or CR<sup>6</sup>R<sup>7</sup>CH<sub>2</sub>) where R<sup>6</sup> is hydrogen and R<sup>7</sup> is selected from the group consisting of the side chains of naturally occurring alpha amino acids, or R<sup>6</sup> and R<sup>7</sup> are independently selected from the group consisting of hydrogen, (C<sub>1</sub>-C<sub>6</sub>)alkyl, aryl, aralkyl, heteroaryl, hydroxy, (C<sub>1</sub>-C<sub>6</sub>)alkoxy, (C<sub>1</sub>-C<sub>6</sub>)alkylthio, NR<sup>3</sup>R<sup>4</sup> and SR<sup>5</sup>, where R<sup>3</sup> and R<sup>4</sup> are as defined below, and R<sup>5</sup> is hydrogen, (C<sub>1</sub>-C<sub>6</sub>)alkyl, hydroxy, (C<sub>1</sub>-C<sub>6</sub>)alkoxy, or (C<sub>1</sub>-C<sub>6</sub>)alkylthio-substituted (C<sub>1</sub>-C<sub>6</sub>)alkyl or R<sup>6</sup> and R<sup>7</sup> taken together complete an alicyclic or heterocyclic system; or C<sup>1</sup>-C<sup>n</sup> is CO, CS, CNR<sup>3</sup>;

each of D<sup>1</sup>-D<sup>n</sup> is (CR<sup>6</sup>R<sup>7</sup>)<sub>z</sub> (preferably CR<sup>6</sup>R<sup>7</sup>, CHR<sup>6</sup>CHR<sup>7</sup> or CH<sub>2</sub>CR<sup>6</sup>R<sup>7</sup>) where R<sup>6</sup> and R<sup>7</sup> are as defined above;

each of y and z is zero or an integer from 1 to 10, the sum y + z being at least 2, preferably greater than 2, but not more than 10;

each of G<sup>1</sup>-G<sup>n-1</sup> is -NR<sup>3</sup>CO-, -NR<sup>3</sup>CS-, -NR<sup>3</sup>SO- or -NR<sup>3</sup>SO<sup>2-</sup>, in either orientation, where R<sup>3</sup> is as defined below;

each of A<sup>1</sup>-A<sup>n</sup> and B<sup>1</sup>-B<sup>n</sup> are selected from (Ia), (Ib) or (Ic) such that:

(Ia): B<sup>1</sup>-B<sup>n</sup> is N and A<sup>1</sup>-A<sup>n</sup> is -CO-(CH<sub>2</sub>)<sub>6</sub>-

(Ib): B<sup>1</sup>-B<sup>n</sup> is N and A<sup>1</sup>-A<sup>n</sup> is -CO-NR<sup>3</sup>-(CH<sub>2</sub>)<sub>2</sub>-

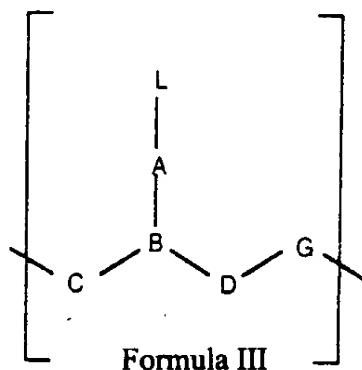
(Ic): B<sup>1</sup>-B<sup>n</sup> is CH and A<sup>1</sup>-A<sup>n</sup> is -NR<sup>3</sup>-CO-(CH<sub>2</sub>)<sub>2</sub>-

Q and I is independently selected from  $-\text{CO}_2\text{H}$ ,  $-\text{CONR}'\text{R}''$ ,  $-\text{SO}_3\text{H}$  or  $-\text{SO}_2\text{NR}'\text{R}''$  or an activated derivative of  $-\text{CO}_2\text{H}$  or  $-\text{SO}_3\text{H}$  and  $-\text{NR}'\text{R}'''$

where R', R'' and R''' are independently selected from the group consisting of hydrogen, alkyl, amino protecting groups, reporter ligands, intercalators, chelators, peptides, proteins, carbohydrates, lipids, steroids, nucleosides, nucleotides, nucleotide diphosphates, nucleotide triphosphates, oligonucleotides, including both oligoribonucleotides and oligodeoxyribonucleotides, oligonucleosides and soluble and non-soluble polymers and as well as nucleic acid binding moieties and

each of x1 and y1 is an integer of from 0 to 10.

107. The method according to claim 86 wherein at least one of said nucleic acid A binding probes comprise at least one monomer subunit of general Formula III



each of L is a ligand independently selected from the group consisting of hydrogen, hydroxy, C<sub>1</sub>-C<sub>4</sub>)alkanoyl, naturally occurring nucleobases, non-naturally occurring nucleobases, aromatic moieties, DNA intercalators, nucleobase-binding groups, heterocyclic moieties, reporter ligands and chelating moieties, wherein at

least one of L is a non-nucleobase electron acceptor or a donor moiety and at least 2 of L being a nucleobase binding group, or a naturally or non-naturally occurring nucleobase;

each of C is  $(CR^6R^7)_y$ , where R<sup>6</sup> is hydrogen and R<sup>7</sup> is selected from the group consisting of the side chains of naturally occurring alpha amino acids, or R<sup>6</sup> and R<sup>7</sup> are independently selected from the group consisting of hydrogen, (C<sub>1</sub>-C<sub>6</sub>)alkyl, aryl, aralkyl, heteroaryl, hydroxy, (C<sub>1</sub>-C<sub>6</sub>)alkoxy, (C<sub>1</sub>-C<sub>6</sub>)alkylthio, NR<sup>3</sup>R<sup>4</sup> and SR<sup>5</sup>, where R<sup>3</sup> and R<sup>4</sup> are as defined below, and R<sup>5</sup> is hydrogen, (C<sub>1</sub>-C<sub>6</sub>)alkyl, hydroxy, (C<sub>1</sub>-C<sub>6</sub>)alkoxy, or (C<sub>1</sub>-C<sub>6</sub>)alkylthio-substituted (C<sub>1</sub>-C<sub>6</sub>)alkyl or R<sup>6</sup> and R<sup>7</sup> taken together complete an alicyclic or heterocyclic system; or C is CO, CS, CNR<sup>3</sup>;

each of D is  $(CR^6R^7)_z$  where R<sup>6</sup> and R<sup>7</sup> are as defined above;

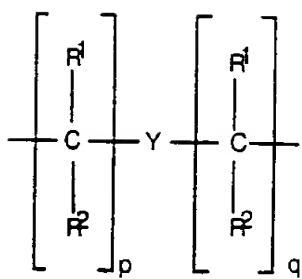
each of y and z is zero or an integer from 1 to 10, the sum y + z being at least 2;

each of G is -NR<sup>3</sup>CO-, -NR<sup>3</sup>CS-, -NR<sup>3</sup>SO- or -NR<sup>3</sup>SO<sup>2-</sup>, in either orientation, where R<sup>3</sup> is as defined below;

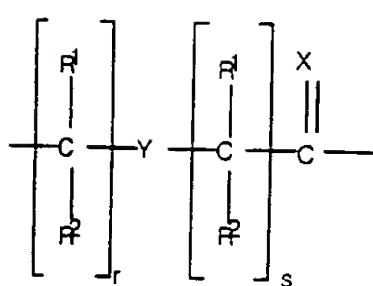
each of A and B are selected such that:

(a) A is a group of formula (I/A), (I/B), (I/C) or (I/D), and B is N or R<sup>3</sup>N<sup>+</sup>; or

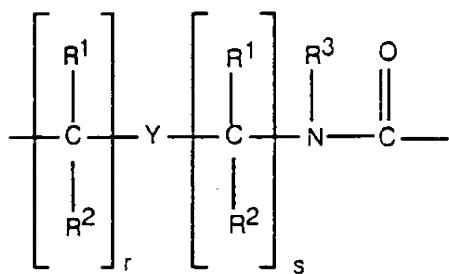
(b) A is a group of formula (I/D) and B is CH;



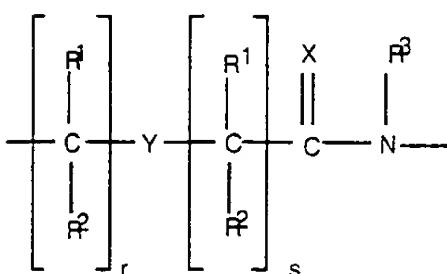
### Formula I/A



### Formula I/B



### Formula I/C



### Formula I/D

wherein:

X is O, S, Se, NR<sup>3</sup>, CH<sub>2</sub> or C(CH<sub>3</sub>)<sub>2</sub>;

Y is a single bond, O, S or NR<sup>4</sup>;

each of  $p$  and  $q$  is zero or an integer from 1 to 5,

each of  $r$  and  $s$  is zero or an integer from 1 to 5,

each R<sup>1</sup> and R<sup>2</sup> is independently selected from the group consisting of hydrogen,

(C<sub>1</sub>-C<sub>4</sub>)alkyl which may be hydroxy- or (C<sub>1</sub>-C<sub>4</sub>)alkoxy- or (C<sub>1</sub>-C<sub>4</sub>)alkylthio-

substituted, hydroxy, (C<sub>1</sub>-C<sub>4</sub>)alkoxy, (C<sub>1</sub>-C<sub>4</sub>)alkylthio, amino and halogen; and

each R<sup>3</sup> and R<sup>4</sup> is independently selected from the group consisting of hydrogen, (C<sub>1</sub>-C<sub>4</sub>)alkyl, hydroxy- or alkoxy- or alkylthio-substituted (C<sub>1</sub>-C<sub>4</sub>)alkyl, hydroxy, (C<sub>1</sub>-C<sub>6</sub>)-alkoxy, (C<sub>1</sub>-C<sub>6</sub>)-alkylthio and amino;

where R', R" and R'" are independently selected from the group consisting of hydrogen, alkyl, amino protecting groups, reporter ligands, intercalators, chelators, peptides, proteins, carbohydrates, lipids, steroids, nucleosides, nucleotides, nucleotide diphosphates, nucleotide triphosphates, oligonucleotides, including both oligoribonucleotides and oligodeoxyribonucleotides, oligonucleosides and soluble and non-soluble polymers and as well as nucleic acid binding moieties and

each of x1 and y1 is an integer of from 0 to 10.

108. The method according to claim 86 wherein said triple stranded complex is more thermostable than a triple stranded complex formed by two nucleic acid A binding probes B binding with one nucleic acid A.

109. A triple stranded complex comprising a nucleic acid A, a nucleic acid A binding probe B, said nucleic acid A binding probe B having a base sequence and a binding region which binds to nucleic acid A, and one or more nucleic acid A binding probe C parts wherein said one or more nucleic acid A binding probe C parts, comprise a base sequence different from the sequence of nucleic acid A binding probe B and a binding region, wherein said binding region, which binds to nucleic acid A, is longer as compared with the binding region of nucleic acid A binding probe B; and wherein said triple stranded complex is more thermostable

than a triple stranded complex formed from either of: (a) two nucleic acid A binding probes B binding with one nucleic acid A; or (b) two of said nucleic acid A binding probes C binding with one nucleic acid A.

110. The complex according to claim 109 containing only one nucleic acid A binding probe C part.

111. The complex according to claim 109 wherein said nucleic acid A binding probe B comprises a binding region of 4 to 10 bases.

112. The complex according to claim 109 wherein said binding region of the one or more nucleic acid A binding probe C parts has a length of at least 6 bases.

113. The complex according to claim 109 wherein said triple stranded complex comprises two different nucleic acid A binding probe C parts.

114. The complex according to claim 113 wherein said two different nucleic acid A binding probe C parts of the triple stranded complex form a binding region which is comprised of two distinct triple helical binding regions, wherein each distinct triple helical binding region is formed from the binding of the two different nucleic acid A binding probe C parts each to a distinct, non-overlapping, region on nucleic acid A.

115. The complex according to claim 114 wherein said two different nucleic acid A binding probe C parts bind juxtaposed on nucleic acid A.

116. The complex according to claim 109 wherein said binding region of nucleic acid A binding probe B comprises only pyrimidine bases but the binding region of the one

or more nucleic acid A binding probe C parts comprises at least one non-pyrimidine base.

117. The complex according to claim 109 wherein said nucleic acid A binding probe B is bound to nucleic acid A via Hoogsteen base pairing and the one or more nucleic acid A binding probe C parts are bound to nucleic acid A via Watson Crick base pairing.
118. The complex according to claim 109 wherein at least one of said nucleic acid A binding probes is labelled and the presence of said label in the triple stranded complex is used for determining the presence or amount of the nucleic acid A.
119. The complex according to claim 109 wherein at least one of said nucleic acid A binding probes is a nucleic acid analogue.
120. The complex according to claim 109, wherein said nucleic acid analogue is a peptide nucleic acid.
121. The complex according to claim 109, wherein said triple stranded complex is more thermostable than a triple stranded complex formed by two nucleic acid A binding probes B binding with one nucleic acid A.
122. A method of forming a triple stranded binding complex comprising reacting a nucleic acid molecule A with a nucleic acid A binding probe B, said nucleic acid A binding probe B having a base sequence and binding region which binds to nucleic acid A, and one or more nucleic acid A binding probe C parts, wherein said one or

more nucleic acid A binding probe C parts comprise a base sequence different from the sequence of nucleic acid A binding probe B and a binding region, wherein said binding region, which binds to nucleic acid A, is longer as compared with the binding region of nucleic acid A binding probe B; wherein said triple stranded complex is more thermostable than a triple stranded complex formed from either of: (a) two nucleic acid A binding probes B binding with one nucleic acid A; or (b) two of said nucleic acid A binding probes C binding with one nucleic acid A.

123. The method according to claim 122 wherein said triple stranded complex contains only one nucleic acid A binding probe C part.
124. The method according to claim 122 wherein said nucleic acid A binding probe B comprises a binding region of 4 to 10 bases.
125. The method according to claim 122 wherein said binding region of the one or more nucleic acid A binding probe C parts has a length of at least 6 bases.
126. The method according to claim 122, wherein said triple stranded complex comprises two different nucleic acid A binding probe C parts.
127. The method according to claim 122, wherein said two different nucleic acid A binding probe C parts of the triple stranded complex form an aggregate binding region which is comprised of two distinct triple helical binding regions, wherein each distinct triple helical binding region is formed from the binding of the two

different nucleic acid A binding probe C parts each to a distinct, non-overlapping, region on nucleic acid A.

128. The method according to claim 127, wherein said two different nucleic acid A binding probe C parts bind juxtaposed on nucleic acid A.

129. The method according to claim 122, wherein said binding region of nucleic acid A binding probe B comprises only pyrimidine bases but the aggregate binding region of the one or more nucleic acid A binding probe C parts comprises at least one non-pyrimidine base.

130. The method according to claim 122, wherein said nucleic acid A binding probe B is bound to nucleic acid A via Hoogsteen base pairing and the one or more nucleic acid A binding probe C parts are bound to nucleic acid A via Watson Crick base pairing.

131. The method according to claim 122, wherein at least one of said nucleic acid A binding probes is a nucleic acid analogue.

132. The method according to claim 122, wherein said nucleic acid analogue is a peptide nucleic acid.

133. The method according to claim 122 wherein said triple stranded complex is more thermostable than a triple stranded complex formed by two nucleic acid A binding probes B binding with one nucleic acid A.

134. A method for determining the presence or amount of a nucleic acid A, comprising: (a) forming a triple stranded complex between said nucleic acid A, a nucleic acid A binding probe B, said nucleic acid A binding probe B having a base sequence and a binding region which binds to nucleic acid A, and two nucleic acid A binding probe C parts, wherein said two nucleic acid A binding probe C parts comprise a base sequence different from the base sequence of nucleic acid A binding probe B and a binding region wherein said binding region, which binds to nucleic acid A, is longer as compared with the binding region of nucleic acid A binding probe B; and (b) determining the presence or amount of said nucleic acid A by measuring for the presence or amount of said triple stranded complex.

135. The method according to claim 134, wherein said two nucleic acid A binding probe C parts of the triple stranded complex are different and form a binding region which is comprised of two distinct triple helical binding regions, wherein each distinct triple helical binding region is formed from the binding of the two different nucleic acid A binding probe C parts each to a distinct, non-overlapping, region on nucleic acid A.

136. The method according to claim 135, wherein said two different nucleic acid A binding probe C parts bind juxtaposed on nucleic acid A.

137. The method according to claim 135, wherein said triple stranded complex is at least six bases in length and each of the two different nucleic acid A binding probe